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Report

# Proteomic Analysis Reveals the Role of Synaptic Vesicle Cycling in Sustaining the Suprachiasmatic Circadian Clock

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## Summary

**The central circadian pacemaker of the suprachiasmatic nucleus (SCN) is characterized as a series of transcriptional/posttranslational feedback loops [1, 2]. How this molecular mechanism coordinates daily rhythms in the SCN and hence the organism is poorly understood. We conducted the first systematic exploration of the "circadian intracellular proteome" of the SCN and revealed that ~13% of soluble proteins are subject to circadian regulation. Many of these proteins have underlying nonrhythmic mRNAs, so they have not previously been noted as circadian. Circadian proteins of the SCN include rate-limiting factors in metabolism, protein trafficking, and, intriguingly, synaptic vesicle recycling. We investigated the role of this clock-regulated pathway by treating organotypic cultures of SCN with botulinum toxin A or dynasore to block exocytosis and endocytosis. These manipulations of synaptic vesicle recycling compromised circadian gene expression, both across the SCN as a circuit and within individual SCN neurons. These findings reveal how basic cellular processes within the SCN are subject to circadian regulation and how disruption of these processes interferes with SCN cellular pacemaking. Specifically, we highlight synaptic vesicle cycling as a novel point of clock cell regulation in mammals.**

## Results

Circadian pacemaking in the suprachiasmatic nucleus (SCN), the principal coordinator of circadian rhythms in mammals, arises from interlocked transcriptional/posttranslational feedback loops [1, 2]. Circadian signaling between SCN neurons and their targets relies upon daily cycles of electrical firing and neurosecretion. How the molecular clock drives these output rhythms is unclear, although transcriptional profiling has identified a number of circadian-regulated genes in the SCN [3, 4], while proteomic approaches have identified extracellular, secretory products of the SCN [5]. Here, we conducted a systematic exploration of the SCN "circadian proteome" via two-dimensional difference gel electrophoresis (2D-DIGE) combined with mass spectrometry (MS) to identify intracellular factors likely to contribute to circadian timekeeping in the SCN. The utility of this approach has been demonstrated in previous analyses of the hepatic circadian proteome [6, 7].

Soluble proteins were displayed by 2D-DIGE with SCN tissue harvested from mice on the second cycle after transfer from 12 hr light and 12 hr dim red light to constant dim red light. Proteins were extracted from two SCN per replicate, with six replicates at each of four circadian times (CT) (CT0, 6, 12, and 18; n = 12 mice per time point; n = 48). Applying this experimental design and previously described criteria [6, 7], 871 protein "spots" were reliably detected across gels (see Figure S1 available online). Of these, 115 exhibited a statistically significant change over circadian time (analysis of variance [ANOVA], $p < 0.05$), and we focused on 53 spots with a clear circadian profile in which abundance changed significantly between samples 12 hr apart (Figure S2). Peak abundances of these spots extended across the cycle, with more peaks during circadian day (65%) than night (35%). We estimate that between 6% (53 of 871) and 13% (115 of 871) of robustly expressed soluble SCN proteins are under circadian regulation. This compares with previous estimates of the SCN circadian transcriptome (~5% of genes assayed [3]), whereas in the liver, 10%–20% of detectable proteins were under circadian regulation [7].

The original gels and two further series of preparative gels from independent SCN samples were analyzed by generating uninterpreted peptide fragmentation data via liquid chromatography followed by tandem mass spectrometry (LC-MS/MS) and by using the Mascot search engine. In several cases, different rhythmic spots represented isoforms of the same protein, but ultimately we identified 34 proteins as circadian regulated (see Table 1 for an annotated list). These represented several functional categories, including cellular metabolism (glutamate dehydrogenase 1, enolase, M2-pyruvate kinase, NADP-dependent isocitrate dehydrogenase, aldolase, 3-oxoacid CoA transferase 1) and heat-shock proteins (heat-shock proteins 1$\alpha$, 8, 9A, and 84 kDa and chaperone subunit 5$\varepsilon$) (Figures 1A and 1B). Significantly, factors associated with synaptic vesicle cycling were also rhythmic, including N-ethylmaleimide-sensitive fusion protein (NSF), the binding protein for NSF (SNAP-$\beta$), two isoforms of synapsin II, and the zeta isoform of the 14-3-3 scaffolding protein (Figure 1C). Whereas other family members, synapsin I and SNAP-25, have mRNAs that cycle in the SCN [4], it was evident that none of our putative circadian proteins have been associated with rhythmic SCN mRNA expression [3, 4]. To cross-validate this unexpected finding, an independent series of SCN samples were subjected to quantitative polymerase chain reaction (PCR). In this sample set, the canonical clock genes *Per1*, *Per2*, and *Rev-erb*$\alpha$ exhibited significant changes over time, as did the circadian output gene *Dbp* (Figures 1D–1F; Table S1).

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Table 1. Circadian Proteins of Suprachiasmatic Nucleus Revealed by DIGE/MS

| Spot Number | Protein ID | | MW | Mascot Score | Unique Peptides |
|---|---|---|---|---|---|
| 367 | 309317 | 84 kDa heat-shock protein | 83638 | 356 | 6 |
| 369 | 6754354 | Heat-shock protein 1, alpha | 85134 | 123 | 2 |
| 573 | 1171774 | Vesicle-fusing ATPase (vesicular-fusion protein NSF) | 83083 | 686 | 15 |
| 638 | 440121 | Immunoglobin gamma chain | 51545 | 156 | 3 |
| 664 | 6754256 | Heat-shock protein 9A | 73768 | 1279 | 22 |
| 680 | 42542422 | Heat-shock protein 8 | 71056 | 1580 | 32 |
| 744 | 599966 | TOAD-64 | 62638 | 740 | 14 |
| 762 | 8567410 | Synapsin II | 52818 | 238 | 4 |
| 797 | 14389431 | Stress-induced phosphoprotein | 63156 | 677 | 20 |
| 920 | 6671702 | Chaperone subunit 5 (epsilon) | 60042 | 582 | 14 |
| 921 | 110625624 | t complex protein | 60867 | 1324 | 21 |
| 937 | 17390900 | Ina protein | 55520 | 756 | 17 |
| 1008 | 6679687 | Protein disulphide isomerase associated 3 | 57042 | 648 | 11 |
| 1011 | 1405933 | M2-pyruvate kinase | 58448 | 993 | 20 |
| 1082 | 8567410 | Synapsin II | 52818 | 291 | 5 |
| 1091 | 13097345 | 3-oxoacid CoA transferase 1 | 56352 | 655 | 10 |
| 1175 | 30931187 | Glutamate dehydrogenase 1 | 61640 | 640 | 12 |
| 1191 | 38328220 | Sept11 protein | 49991 | 124 | 3 |
| 1279 | 13097429 | Methionine adenosyltransferase II, alpha | 44003 | 169 | 3 |
| 1326 | 54114937 | Eno1 protein | 50165 | 540 | 11 |
| 1363 | 15929689 | Creatine kinase | 42985 | 249 | 5 |
| 1408 | 27370092 | Tu translation elongation factor, mitochondrial | 44003 | 169 | 3 |
| 1506 | 3631400 | NADP-dependent isocitrate dehydrogenase | 47030 | 535 | 12 |
| 1622 | 160298209 | Glutamate oxoloacetate transaminase 1 | 42232 | 602 | 11 |
| 1644 | 7548322 | Aldolase A | 39925 | 599 | 13 |
| 1647 | 7548322 | Aldolase A | 39925 | 659 | 13 |
| 1656 | 7548322 | Aldolase A | 39925 | 648 | 10 |
| 1864 | 54611730 | NSF attachment protein beta | 33878 | 361 | 7 |
| 1901 | 13097375 | Electron transferring protein | 35360 | 230 | 5 |
| 2043 | 1841387 | 14-3-3 zeta | 27879 | 444 | 8 |
| 2071 | 12805413 | Eschs1 protein | 31636 | 258 | 6 |
| 2079 | 6755198 | Proteasome subunit, alpha type 6 | 27811 | 258 | 5 |
| 2210 | 28436836 | Triosephosphate isomerase 1 | 27038 | 471 | 12 |
| 2147 | 94371142 | Predicted: similar to glutathione S-transferase | 25864 | 264 | 5 |
| 2216 | 21465838 | Chain C, guanine nucleotide exchange region of intersectin | 21189 | 105 | 2 |
| 2327 | 53237015 | Peptidylprolyl isomerase A | 18131 | 312 | 7 |

Spot number refers to arbitrary numbering of features within the 2D gel field recognizable throughout the study.

Expression of *Cry1* failed narrowly to reach significance by ANOVA. Of the 21 genes encoding putative circadian proteins tested in this way, only 3 (11%) showed significantly circadian mRNA expression in the SCN (Figures 1D–1F; Table S1). This contrasts with significant circadian expression exhibited by 8 of the 18 (44%) of these genes expressed in the liver (data not shown). To probe further for low-amplitude mRNA rhythms, we reanalyzed the data by cross-correlation to a 24 hr sinusoid (see Supplemental Experimental Procedures), which revealed a statistically significant cycle in *Cry1* expression (Table S1) and 8 (38%) of the other 21 genes. Hence, depending on the analysis used, between 11% and 38% of the rhythmic proteins are accompanied by significantly rhythmic mRNA. As with the liver, where ~50% of rhythmic proteins were accompanied by nonrhythmic mRNAs [7], we conclude that posttranscriptional regulation is a significant feature in circadian sculpting of the SCN proteome.

Circadian regulation of metabolic factors parallels the well-characterized circadian cycle of glucose utilization in the SCN [8]. Circadian modulation of vesicle and membrane recycling proteins, however, was unprecedented. We therefore explored the functional significance of these clock-regulated factors in circadian pacemaking. Synapsins are neuron-specific phosphoproteins exclusively associated with small synaptic vesicle membranes and actin filaments at the synaptic terminal. Synapsin II controls the reserve pool of synaptic vesicles [9] and has been implicated in the etiology or pathogenesis of schizophrenia [10]. The 14-3-3ζ adaptor protein associates with various neural signaling pathways [11] and again regulates exocytosis by controlling the releasable vesicle pool [12]. The ATPase NSF directs the formation of soluble NSF attachment protein receptor (SNARE) complexes, initiating endocytosis of fused synaptic vesicles from the presynaptic membrane and vesicle fusion preceding neurotransmitter release [13]. The binding of NSF to SNARE complexes is facilitated by NSF attachment proteins (SNAPs) [14], the beta isoform being brain specific and, as with NSF, implicated in both exocytosis and endocytosis [15]. In the SCN, NSF and synapsin II were coregulated with comparable circadian profiles and peak abundance during circadian day, whereas levels of 14-3-3ζ and SNAP-β were lowest at CT12 (Figure 1C). We confirmed SCN expression of NSF and synapsin by immunocytochemistry and western blots, with clear punctate signals evident by immunostain and single (NSF) and double (synapsin) bands by western blot. Despite exhaustive testing, however, we could detect no temporal change in expression (Figure S3). This might arise if multiple isoforms were expressed, only some of which were clock regulated. We therefore performed 2D-DIGE/MS on a further series of samples to identify potential nonrhythmic isoforms, evident, for example, as a charge train of adjacent spots. This revealed a second NSF spot (571), which was not statistically rhythmic and had a profile different from that of the circadian isoform (spot 573, Figures 2A and 2D). The nonrhythmic isoform appeared at the same molecular weight but was more acidic than its counterpart, indicating posttranscriptional or post-translational processing. Similarly, three further isoforms of synapsin II were identified, none of which exhibited significant effects of time but each of which were nonetheless expressed at peak levels later than the rhythmic spots (Figures 2B, 2C, and 2E). One nonrhythmic isoform had a similar molecular weight to its rhythmic counterpart but had an increased pI (Figure 2B). A larger rhythmic isoform also had two nonrhythmic counterparts of similar molecular weight, one more acidic, the other more basic (Figure 2C). These results emphasize the temporal complexity of synaptic vesicle protein expression in the SCN arising from circadian transcriptional/posttranslational processing. They also reinforce the value of

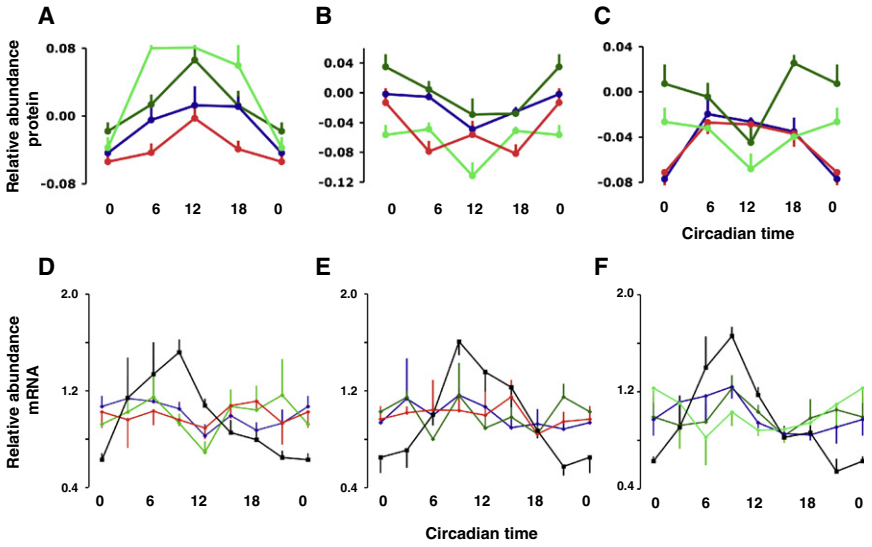


Figure 1. Circadian Metabolic and Vesicle-Cycling Proteins in Suprachiasmatic Nucleus Identified by DIGE/MS Are Accompanied by Non-circadian mRNA Expression

(A–C) Representative plots of significantly rhythmic proteins with peak phases at either (A) circadian dusk (red, synapsin II [spot 762]; blue, glutamate dehydrogenase; dark green, sept 11 protein; light green, stress-induced phosphoprotein) or (B) circadian dawn (red, neuronal internexin; blue, chaperone subunit 5; dark green, t complex protein; light green, heat-shock protein 8) and (C) proteins involved in synaptic vesicle cycling (red, synapsin II [spot 1082]; blue, N-ethylmaleimide-sensitive fusion protein [NSF]; dark green, NSF attachment protein; light green, 14-3-3ζ).

(D–F) Circadian expression of mRNAs encoding canonical clock factors but absence of circadian expression of mRNAs encoding rhythmic proteins in suprachiasmatic nucleus (SCN): (D) black = *Per1*, red = *neuronal internexin*, blue = *chaperone subunit 5*, green = *heat-shock protein 8*; (E) black = *Per2*, red = *synapsin II*, blue = *NSF*, green = *NSF attachment protein*; (F) black = *Dbp*, blue = *glutamate dehydrogenase*, dark green = *sept 11 protein*, light green = *stress-induced phosphoprotein*. All data plotted as mean + standard error of the mean (SEM); n = 3–6 per time point; CT0 replotted for clarity.

2D-DIGE/MS in identifying individually rhythmic isoforms that would otherwise be overlooked.

To investigate the role of synaptic vesicle cycling in cellular and circuit pacemaking, we treated organotypic SCN slices with either dynasore [16], a small-molecule inhibitor of dynamin and hence an inhibitor of endocytosis, or botulinum toxin A (Botox A), an inhibitor of exocytosis [17]. Inevitably, by interfering with one phase of vesicle cycling, these treatments would ultimately perturb both. Therefore, by comparing the effects of Botox A and dynasore, we hypothesized that we would be able to reveal the global contribution of vesicle cycling to the SCN clockwork. Circadian gene expression across the SCN and within individual SCN cells was monitored by three independent optical reporters [18]. The *mPer1::dsEGFP* transgene provides a fluorescent signal of transcriptional activation of the *Per1* gene [19], whereas the *mPer1::luciferase* transgene provides a bioluminescent report of *mPer1* activation [20]. Tissues from the *mPer2::luciferase* knockin mouse provide a bioluminescent report for Per2 protein cycling [21]. Previous characterization has confirmed that, in the SCN, these signals are neuronal rather than glial in origin.

Vehicle had no effect on circadian gene expression in SCN slices, whereas dynasore significantly reduced the amplitude of *mPer1::dsEGFP* fluorescence to approximately one-third that of control (Figure 3A; Figure 4A). Video microscopy showed that the effect of dynasore was evident in individual neurons across the SCN (Figure 4B). Dynasore also inhibited circadian transcription in *mPer1::luciferase* slices, where the baseline level of bioluminescence immediately fell and the amplitude of oscillation was reduced to ~50% of control. This effect was reversible, the oscillation in *mPer1*-driven bioluminescence being restored on washout (Figure 3B). Treatment with dynasore also caused significant damping in the rhythm of Per2 protein expression by ~30% (Figure 3C

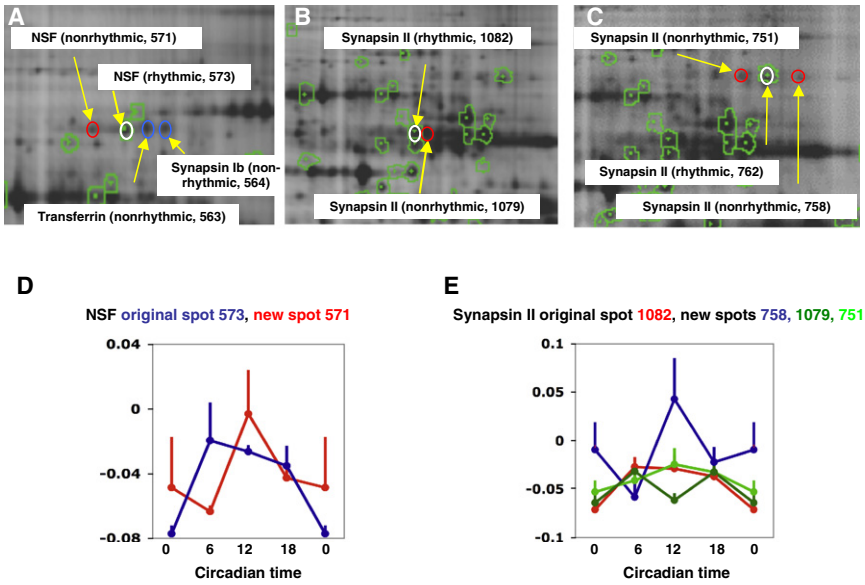


Figure 2. DIGE/MS Reveals Multiple Isoforms of NSF and Synapsin in SCN

(A–C) Magnified views of two-dimensional difference gel electrophoresis (2D-DIGE) gels showing (A) NSF isoforms and (B) isoforms of synapsin II at approximately 58 kDa and (C) isoforms of synapsin II at approximately 86 kDa. Spots marked with a red circle are nonrhythmic.

(D and E) Circadian profiles of rhythmic and nonrhythmic isoforms of NSF and synapsins identified by DIGE/mass spectrometry (MS) (mean + SEM; CT0 replotted for clarity).

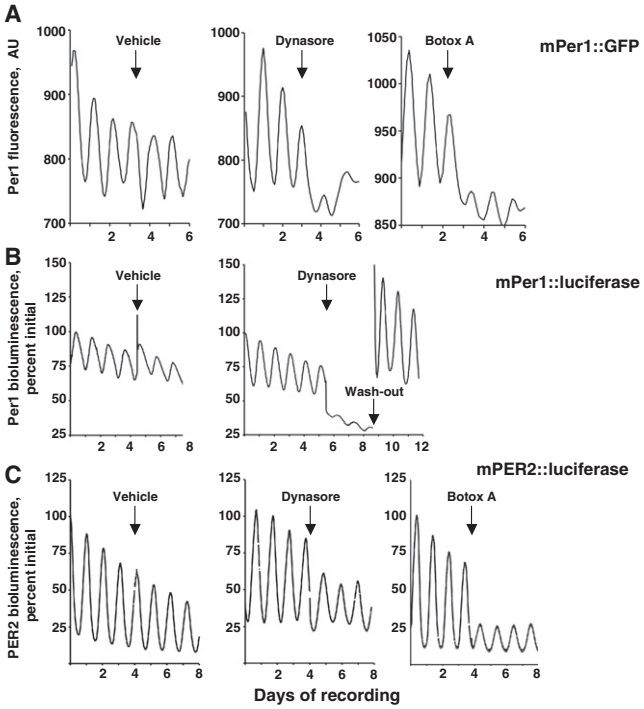


Figure 3. Compromised Circadian Pacemaking in Organotypic SCN Slices in which Vesicle Recycling Is Dysregulated by Treatment with Dynasore or Botox A

Representative recordings of circadian gene expression from SCN slices carrying (A) *mPer1::EGFP*, (B) *mPer1::luciferase*, or (C) *mPer2::luciferase* reporter genes, treated with either vehicle (arrow, left), dynasore (80 μM, center), or Botox A (6.7 nM, right), monitored by (A) video-fluorescence microscopy or (B and C) photomultiplier assembly.

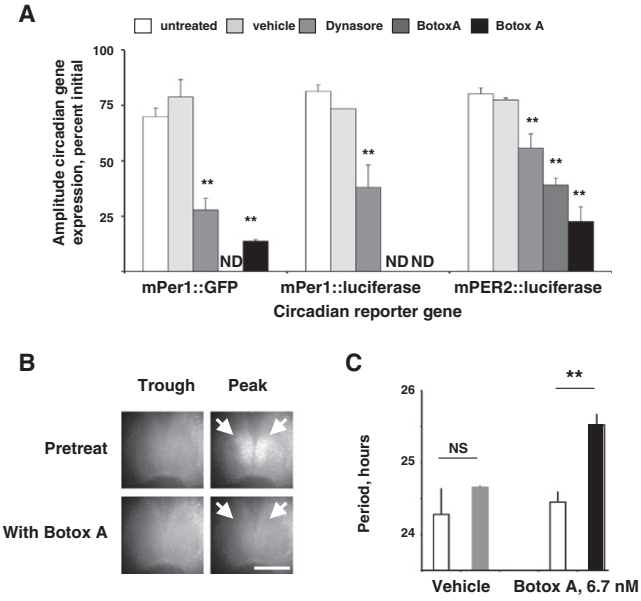


Figure 4. Acute Inhibition of Endocytosis and Exocytosis Damps Circadian Gene Expression and Prolongs Circadian Period in Organotypic SCN Slices

(A) Group data reveal significant suppression of circadian gene expression cycles in SCN treated with dynasore (80 μM) or Botox A (dark gray 6.7 nM, black 67 nM). All data plotted as mean + SEM; n = 3–7; ND = not determined.

(B) Representative fluorescence images of *mPer1::EGFP* SCN slices before and after treatment with Botox A (67 nM) reveal loss of peak signal (arrow, scale bar represents 500 μm).

(C) Circadian period of bioluminescent gene expression rhythms in SCN (mean + SEM) before (white columns) and after treatment with either vehicle (gray column) or Botox A (black column). **$p < 0.01$ versus untreated control condition; NS = not significant.

and Figure 4A). Hence, circadian gene expression in the SCN pacemaker, as monitored by three independent reporters, was disrupted by interference with vesicle cycling.

Botox A had an even more dramatic effect upon circadian pacemaking. It acutely suppressed the amplitude of oscillation by 50%–75%, both across the SCN as a whole and within individual SCN neurons monitored with either *mPer1::dsEGFP* or *mPer2::luciferase* reporters (Figure 3; Figure 4A). Botox A also lengthened the period of oscillation by approximately 1 hr (two-way ANOVA drug versus time interaction $p = 0.038$) (Figure 4C). Furthermore, the relative amplitude error of *mPer2::luciferase* emission was increased, reflecting less precise circadian control over Per2 protein expression (Botox A pretreatment = 0.033 ± 0.005, posttreatment = 0.051 ± 0.009; mean ± standard error of the mean [SEM]), whereas in vehicle-treated slices, the amplitude error declined (vehicle pretreatment = 0.047 ± 0.009, posttreatment = 0.023 ± 0.003; two-way ANOVA interaction drug versus time $p = 0.024$). This long-term compromise to pacemaking was evident from video analysis of cellular bioluminescence rhythms (Figure 5A), which showed that the reduced amplitude of the SCN signal and its increased error were associated with a reduction in the amplitude of pacemaking within individual cells (Figure 5B) plus an accompanying loss of phase coherence of oscillations between individual SCN neurons (Figure 5C). This was reflected by a decrease in the mean vector in the Rayleigh test, measured after three weeks of treatment (vehicle control 0.963 ± 0.008, Botox A 0.605 ± 0.152, t test, $p = 0.04$; n = 3 slices for both groups, 30–32 cells per slice). Thus, disruption of the endocytosis/exocytosis vesicle cycle compromised SCN circadian pacemaking.

## Discussion

We have revealed clock-regulated SCN factors not previously identified by transcriptional profiling, principally because their abundance is regulated at stages beyond the level of mRNA expression. The extent of circadian regulation (~6%–13% of detectable spots) was greater than in pineal gland [22] and retina [23] (3.4% and 2.7%, respectively). This likely reflects our more sensitive analysis [6] and is comparable to that in the liver [7], highlighting a common role for the circadian clock in posttranscriptional regulation.

Our exploration of the intracellular proteome of the SCN is different from, and complements, that of Hatcher et al. [5], which characterized extracellular factors released from electrically stimulated SCN explants. This revealed neuropeptides implicated in intercellular signaling, and one derived from the pro-SAAS precursor [24], which phase shifted the electrical cycle of SCN explants, indicative of a role in retinally mediated entrainment. Our screen identified rhythmic metabolic enzymes and factors involved in synaptic vesicle cycling. This temporal orchestration of protein expression provides an efficient means of sustaining the well-characterized daytime surge in SCN metabolic rate and electrical firing [25]. For example, our recordings reveal a circadian cycle of spontaneous electrical firing in *mPer2::luciferase* slices, ranging between 7.4 ± 1.8 Hz (mean ± SEM, circadian day) and

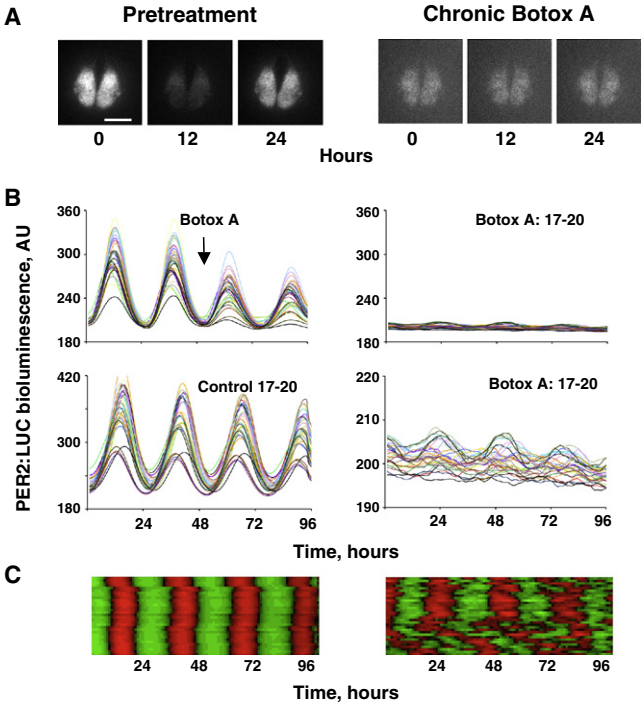


Figure 5. Chronic Inhibition of Exocytosis by Botox A Suppresses and Desynchronizes Cellular Circadian Pacemaking in the SCN

(A) Representative bioluminescence images of SCN slice immediately before and after 17 days of treatment with 6.7 nM Botox A. Scale bar represents 500 μm.

(B) Bioluminescence rhythms from individual cells recorded in slice (A) show immediate damping (upper left, 47.9% +3.4%) and chronic suppression (upper right). Expansion of the ordinate (lower right) reveals phase dispersal of weakly rhythmic cells, which contrasts with the coherent cellular organization observed in control, vehicle-treated slices (lower left).

(C) Raster plots of cells depicted in (B) subject to vehicle (left) or chronic Botox A (right).

2.4 ± 0.7 Hz (circadian night). This electrical rhythm is phase locked to the bioluminescence rhythm (M.H.H., S.R. Williams, and S.E. Atkinson, unpublished data) and will, inevitably, be underpinned by increased rates of vesicle recycling in the day and a decrease during electrically quiescent circadian night. Rhythmic expression of these factors also highlights their potential roles as more general regulators of neuronal functions. For example, the rhythm in NSF within the SCN is comparable to its rhythmic expression in the retina [23], whereas induction of sexual behavior by estrogen is associated with upregulation of NSF in the hypothalamus [26]. Changes in NSF in the SCN are accompanied by alterations in the NSF regulator SNAP, alongside 14-3-3ζ and synapsin II, all indicative of a coordinated circadian program controlling synaptic vesicle cycling.

The importance of vesicle cycling in the pacemaker is further highlighted by the *earlybird* mutation in the GTPase Rab3 [27]. Rab3 regulates synaptic vesicle transport and is essential for the regulation of $Ca^{2+}$-triggered vesicle release probability [28]. The *earlybird* mutation reduces protein levels and dramatically accelerates the clock by ~2 hr. Our biochemical study complements this genetic analysis and focuses on events within the active zone of the pre- and postsynaptic membranes. Inhibiting either exocytosis or endocytosis to compromise vesicle cycling suppressed the amplitude of circadian gene expression. Although it is possible that off-target actions of dynasore or Botox A may contribute to this diminution by, perhaps, compromising synthesis of GFP or luciferase, we consider this unlikely because interference with both axes of the vesicle cycle had the same effect, and dynasore does not affect overall rates of protein synthesis [16]. Moreover, the long-term effects of Botox A in lengthening circadian period and decreasing cellular coherence within the SCN circuit would not arise from suppression of reporter synthesis per se.

Contrary to earlier views of SCN neurons as robust, cell-autonomous clocks, circuit-level properties are essential features of pacemaking. They defend the clock against genetic perturbation [29], and pharmacological blockade of action-potential firing leads to loss of phase coherence and amplitude of circadian gene expression [20, 30]. More specifically, signaling by the neuropeptide vasoactive intestinal peptide (VIP) via the VPAC2 receptor is essential for synchronized and high-amplitude cellular pacemaking [18, 31]. Synaptic vesicle cycling will contribute to this signaling in two ways. First, presynaptic mechanisms will sustain the release of VIP (and other factors) onto postsynaptic targets. Second, postsynaptic processes will determine the endocytosis, trafficking, and recycling of the activated G protein-coupled VPAC2 receptor. This receptor signals through adenylyl cyclase (AC), and we have shown that inhibition of AC in the SCN phenocopies the VPAC2 null mutation [32]. The rates and efficiencies of these pre- and postsynaptic processes will therefore influence the dynamics of VIP/VPAC2 signaling. Thus, dynasore or Botox A will dysregulate VIP-ergic (and other) signaling, disorganizing the pacemaker circuit. Conversely, under normal circumstances, circadian expression of vesicle regulatory factors represents a positive adaptation to sustain electrical firing and circuit function across the circadian cycle.

In addition to this circuit level role, we cannot rule out an intracellular action, in which synaptic recycling itself feeds back onto the molecular oscillator. Disruption of the endocytic cycle by the *shibire*$^{ts}$ (*shi*$^{ts}$) mutation of dynamin within the pacemaker cells of *Drosophila* lengthens the period of behavioral rhythms and reduces levels of the positive circadian transcription factor CLOCK within the core feedback loops [33]. These effects can be modulated by manipulating the endocytic factors clathrin light chain and Rab5. Moreover, two transcripts encoding additional components of the endocytic pathway, syndapin and beta-adaptin, are rhythmic [34], supporting the speculation [33] that circadian cycling of endocytosis amplifies intercellular communication and perhaps defines a further feedback loop in the clockwork. Finally, the *Drosophila* homolog of Hsp8 (a circadian factor in the SCN) is hsc70-4 and has been implicated in neurotransmitter exocytosis [35] and clathrin-coated endocytosis, where it interacts genetically with *shibire* [36]. Notwithstanding whether the effects of disrupting endocytosis/exocytosis arise from perturbations of inter- or intracellular signaling, in uncovering circadian rhythms in the expression of factors regulating synaptic vesicle recycling and demonstrating that disruption of these pathways compromises circadian gene expression, we have revealed a new avenue of regulation within the SCN pacemaker.

### Supplemental Data

Supplemental data include Supplemental Experimental Procedures, three figures, and one table and can be found online at http://www.cell.com/current-biology/supplemental/S0960-9822(09)01843-0.

**Acknowledgments**

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